REMARKS

The Examiner rejected claims 9 and 12-23 on the basis that the specification was not enabling for detection of soluble ErbB1 in all biological samples. Claim 9, as amended, clarifies that the invention can be used to detect both soluble and other forms of ErbB1 in biological samples.

The Examiner further rejected claims 9-17 as being unpatentable over Harvey et al., U.S. patent 6,674,753, 1997, Partanen et al. (J. Occup. Med., 1994, vol. 36 pp 1324-1328) or Witters et al. (Clin. Cancer Res., 1995, vol. 1, pp. 551-557) in view of Graus-Porta et al. or Olayioye et al., and further in view of WO 94/11734 (Johansen et al., 1994).

Partanen et al., Witters et al., and Harvey '753 teach the detection of soluble EGFR forms in serum, urine, blood and plasma of cancer patients and teach that increased sEGFR concentrations are associated with disease, and in particular, cancer. In contrast, Applicants'invention teaches that decreased sEGFR concentrations are associated with cancer. Because EGFR is overexpressed in many human cancers and proteolytic cleavage has been believed to release soluble EGFR molecules from the plasma membrane of such tumors, the teachings of the present invention that serum sEGFR concentrations are lower in cancer patients is not obvious. In addition, the immunoassays taught by Partanen et al. and patent '753 which use different antibodies toward EGFR and processes give disparate results for serum sEGFR concentrations compared to those of the present invention. In particular, Baron et al. (J. Immunol. Methods, 1998, vol. 219, pp. 23-43) and Baron et al. (Cancer Epidemiol. Biomark. Prev., 2001, vol. 10, pp. 1175-1185) compared to the immunoassays reported by Partanen et al.

and patent '753 and noted that Applicants' invention has a greater dynamic range of serum sEGFR concentrations in human serum and thus has a superior ability or sensitivity to discern healthy subjects from cancer patients. The immunoassay of the present invention thereby fulfills a long-felt need in the field for an immunoassay having the operational characteristics to test whether sEGFR has utility in the risk assessment, screening, diagnosis, and prognosis of human cancer.

In short, one of ordinary skill would not know that decreased sEGFR concentrations were useful, and that the combination of matter, chemistry, and process (microtiter plates with covalent-linkage, antibodies, buffers, and acridinium-based photochemistry) described by the present invention would yield an immunoassay with unexpectedly superior properties with utility.

The Examiner noted that none of the first three references use the antibodies used by Applicants nor do they use the assay technique used by Applicants. The Examiner further notes that neither Graus-Porta nor Olayioye teach the assay method used by Applicants. Although Graus-Porta and Olayioye teach that two anti-EGFR antibodies, i.e., ERFR1 and 528, can be used for combined immunoprecipitation and immunoblotting purposes, they do not teach that these antibodies can be used to quantify EGFR or soluble ErbB1 in any context, let alone in human body fluids using sandwich-type microtiter plate-based immunoassays

Finally, Johansen does not teach direct labeling with acridinium nor the detection of the EGF receptor. Johansen teaches that chemiluminescent probes, such as acridinium esters, can be used to label avidin/streptavidin complexes in magnetic particle-basesd immunoassays, but does not teach that acridinium esters can be used to directly label any antibodies, and more specifically do not teach labeling antibodies that are specific for AGFR/ErbB1 (including antibody 528).

Moreover, it is not obvious from Johansen that such acridinium-labeled antibodies would retain their bioactivity such that they could support microtiter plate-based immunoassays. In short, because one skilled in the art would not have thought to use an acridinium-coupled anti-EGFR antibodies, particularly acridinium-labeled antibody 528, to measure ErbB1 in human body fluids. Because the references do not use the assay technique described by Applicants, it would not have been obvious to combine the teachings of the three sets of disclosures. Nothing in any of the cited references suggest or teach such a combination and in fact such references teach away from such a combination, e.g., measurement of increased ErbB1 versus Applicants' invention which teaches measurement of decreased ErbB1.

CONCLUSION

Applicants respectfully submit that the present invention is not obviated by the teachings and that the patent application and claims therein, as amended, are in a condition for allowance. Reconsideration is, therefore, respectfully requested.

Respectfully submitted,

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MARKED UP SPECIFICATION

Page 1, lines 7-11:

[The disclosed invention was made with the support of grants from the National Institutes of Health grants 1RO1 CA57534 (Truncated *c*-erbB Receptors in Women with Ovarian Cancer) to Nita J. Maihle, CA09441-13 (Multidisciplinary Basic Research Training in Cancer) to the Mayo Foundation, and 1KO7 CA76170-01A1 (Soluble ErbB1 molecules as Tumor Biomarkers) to Andre T. Baron.]The disclosed invention was made with the support of grants from the National Institutes of Health. The U.S. Government has certain rights in the invention.

Page 1, lines 15-21:

The present invention relates to the discovery of soluble isoforms of an Epidermal Growth Factor Receptor, or sErbB1 variants, the nucleic acid sequences encoding these isoforms, purified recombinant proteins, novel antibodies specific for these isoforms, and the use of immunoassay and other protein assay techniques to measure the concentration of these protein isoforms in a patient biological sample in the femtomolar range. [The present invention also provides diagnostic methods for determining the presence of an ovarian carcinoma in the patient by assaying the concentration of soluble ErbB1 variants in a biological sample from a patient.]The present invention also provides diagnostic methods for assessing the risk of ovarian cancer or for determining the presence of an ovarian carcinoma in the patient by assaying the concentration of soluble ErbB1 variants in a biological sample from a patient.

Page 1, lines 24-25, and page 2, lines 1-7:

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein encoded by the [*c*-erbB1]EGFR/ERBB1 proto-oncogene. Sequence analysis demonstrated that the human EGFR

is the cellular homolog of the *v-erbB* oncogene from the avian erythroblastosis retrovirus [(Downwald](Downward *et al.*, Nature, 307, 521 (1984); Ullrich *et al.*, Nature 309, 418 (1984). In recent years, a series of *c-erbB* related cell surface receptor tyrosine kinases [has]have been identified. The four members of the [*c-erbB*]ERBB proto-oncogene family are: ErbB1/EGFR, ErbB2/lNeu (Coussens *et al.*, Science, 230, 1132 (1988); ErbB3 (Kraus *et al.*, Proc. Nat'l. Acad. Sci. USA, 86, 9193 (1989)); Plowman, *et al.*, Proc. Nat'l. Acad. Sci. USA 87, 4905 [(1991)](1990)); and ErbB4 (Plowman *et al.*, Proc. Nat'l. Acad. Sci. USA, [20]90,1746 (1993)).

Page 2, lines 8-25:

The [ErbB1/EGFR]Epidermal Growth Factor Receptor (EGFR/ErbB1) includes three functional domains: an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The extracellular domain can be further divided into four subdomains (I-IV), including two cysteine-rich regions (II and IV) and two flanking regions (I and III) [(Maihle *et al.*, Proc. Nat'l. Acad. Sci., 88, 1825 (1991)](Lax *et al.*, Cell Regul. 2, 337 (1991)). Subdomains I and III are involved in ligand binding. Ligand binding to the receptor represents the first event in a complex cascade culminating in DNA synthesis and cell division. The full-length 170 kD human EGFR is encoded by two alternatively spliced transcripts of 5.8 and 10.5 kb (Ullrich *et al.*, *supra*). In addition, alternatively spliced mRNA's from the EGFR/ERBB1 gene also encode soluble forms of this receptor. A truncated 80 kD EGFR present in human placenta extracts may be encoded by an alternatively spliced 1.8 kb RNA transcript (Ilekis *et al.*, Mol. Reprod. Devel., 41, 149 (1995)). Furthermore, soluble EGF receptors arise from aberrant transcription products in carcinoma derived cell lines, as exemplified by the epidermoid carcinoma line, A431 (Ullrich *et al.*, *supra*). In this cell line, the EGFR gene is

amplified and rearranged, and a 2.8 kb transcript arises from a translocation between the 5'-region of the EGFR gene and an unidentified region of genomic DNA (Ullrich *et al.*, *supra*; Merlino *et al.*, Mol. Cell. Biol., 5, 1722 (1985); Hunts *et al.*, Somat, Cell Mol. Genet., 11, 477 (1988)). Alternatively spliced [*c*-erbB1]EGFR/ERBB1 RNA transcripts of approximately [2.6-2.7]1.8-2.8 kb that encode secreted, truncated receptors containing only the extracellular ligand binding domain are also found in normal [chicken and rat tissue]human, chicken, rat, and mouse tissues (Maihle *et al.*, [supra]Proc. Nat'l Acad. Sci. USA, 88, 1825 (1991); Petch *et al.*, Mol. Cell. Biol., 10, 2973 (1990); Flickinger *et al.*, Mol. Cell Biol., 12, 883 (1991); Das *et al.*, Endocrinology 134,971 (1994); Rho *et al.*, Mol. Carcinogenesis 11, 19 (1994); Reiter and Maihle, Nucl. Acids Res., 24, 4050 (1996); Tong *et al.*, Endocrinology 137, 1492 (1996)).

Page 3, lines 1-13:

Soluble forms of [the ErbB family of receptors have been]ErbB receptors (sErbB) are being investigated in connection with several cancers (McKenzie, Biochim. Biophys. Acta, 1072, 193 (1991); Brandt-Rauf, Mutat. Res., 333, 203 (1995)). Immunoassay studies show that sErbB2 proteins are elevated in serum samples of patients with breast and ovarian cancer (Mori *et al.*, Jpn. J. Cancer. Res., 81, 489 (1990); Meden *et al.*, Anticancer Res., 17, 757 (1997)). Recent studies suggest that low pretreatment serum sErbB2 levels are positive predictors of responsiveness to hormonal therapy for patients with metastatic breast cancer (Hayes *et al.*, Breast Cancer Treat., 14, 135, (1993); Leitzel *et al.*, J. Clin. Oncol., 13, 1129 (1995); Yamauchi *et al.*, J. Clin. Oncol., 15, 2518 (1997)). Meden *et al.* (*supra*) have reported a positive association between elevated serum p105 sErbB2 levels and shorter survival for patients with stage I through IV epithelial ovarian cancer (EOC). In addition, U.S. Patent No. 5,674,[763]753, issued October 7, 1997, described the use of antibodies against the external EGF binding domain of EGF

receptors to diagnose neoplastic diseases correlated with an increase in the level of an EGF receptor in a patient's blood.

Page 4, lines 3-10:

Thus, the current research concerning the biological role and function of EGF receptors has been contradictory, and does not provide a clear indication of how any particular EGF receptor can be used as a tool in diagnosing any particular cancer type. Therefore, a need exists for the isolation of soluble epidermal growth factor receptor protein molecules and their isoforms, and for the characterization of their correlated disease conditions. In addition, a useful, quantitative diagnostic method to detect the presence of biologically relevant isoforms for diagnosing the onset and progression of diseases associated with these soluble [endothelial]epidermal growth factor receptor protein molecules is also needed.

Page 4, lines 13-25, and Page 5, lines 1-6:

In a first aspect, the invention provides several novel isolated and purified non-genomic nucleic acids which encode soluble isoforms of the human [EGFR ErbB1]EGFR/ErbB1, as well as nucleic acids encoding engineered variants of these proteins. Preferred embodiments of this aspect of the invention are nucleic acid sequences which specifically encode a soluble form of a human EGFR ("sEGFR") whose amino acid sequence comprises the sequence of SEQ ID NO:1. The nucleic acid embodiments of the invention include, *e.g.*, DNA SEQ ID NO:2, which is the naturally occurring sequence encoding the polypeptide SEQ ID NO:1. The nucleic acids of the invention also include nucleic acid sequences which are complementary to or synonymous with SEQ ID NO. 2, (i.e., also encode DNA SEQ ID NO. 1.) Other preferred embodiments of this aspect of the invention include nucleic acids which encode proteins which comprise a sequence which is has at least 90% identity with SEQ ID NO. 1, more preferably at least 95% identity with SEQ ID NO. 1, more preferably at least 98% identity with SEQ ID NO. 1, and most preferably at least 99% identity with SEQ ID NO. 1. A preferred embodiment of these nucleic acids would be

a nucleic acid encoding the naturally occurring truncated variant SEQ ID NO. 3, which is approximately 90.9 % identical to SEQ ID NO. 1 (641 out of 705 amino acids). Other preferred embodiments of these nucleic acids include nucleic acids encoding the point-mutation proteins SEQ ID NO. 4, SEQ ID NO. 5, and SEQ ID NO. 6, which are approximately 99.8% identical to SEQ ID NO. 1 (704 out of 705 amino acids). Other embodiments of the nucleic acid sequence invention include nucleic acids which are complementary to the above nucleic acids.

Page 5, lines 19-25, and Page 6, lines 1-8:

Yet another aspect of the present invention are polypeptides useful for generating antibodies specific to the proteins encoded by the nucleic acid sequences of the invention, including SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, and SEQ ID NO. 6. The polypeptides of the invention comprise an amino acid sequence of 10 to 25 amino acids in length, more preferably 11 to 21 amino acids in length, and most preferably 14 to 20 amino acids in length, wherein the amino acid sequence is identical to an amino acid sequence of similar length in an amino acid sequence selected from the group consisting of: amino acids 628-705 of SEQ ID NO. 1, amino acids 628-705 of SEQ ID NO. 4, amino acids 628-705 of SEQ ID NO. 5, and amino acids 628-705 of SEQ ID NO. 6. Another aspect of the invention is an immunogenic conjugate comprising one of the above polypeptides and a carrier molecule. Preferred carrier molecules for use in immunogenic conjugates include keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Yet another aspect of the present invention are monoclonal or polyclonal antibodies produced using the above polypeptides which [is]are specific for a protein selected from the group consisting of: SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, and SEQ ID NO. 6; and which do not cross-react with other ErbB1 isoforms.

Another aspect of the present invention is an expression cassette comprising: a preselected DNA segment that is complementary to SEQ ID NO:2 that is operably linked to a promoter functional in a host cell. Thus, the present invention provides an expression cassette that

expresses an "antisense" mRNA transcript of a DNA sequence of the invention. Another aspect of the invention is a method of using this transcript by transforming a host cell with an expression cassette comprising the complementary sequence which is expressed within the host cell, and thus altering EGFR and/or sEGER expression, cell growth and/or differentiation of the host cell. In addition, such complementary transcripts may be utilized in RNAse protection assays to determine the level of cellular expression of mRNAs encoding SEQ ID NO. 1.

Page 6, lines 18-25, and Page 7, lines 1-11:

Yet another aspect of the invention is a sandwich immunoassay method for detecting or determining the concentration of soluble and full-length human epidermal growth factor receptor in a biological sample obtained from a patient. The method comprises: a) contacting an amount of a first purified antibody that specifically reacts with a first epitope of the extracellular ligand binding domain of sErbB1 with the patient biological sample to be tested, wherein the first purified antibody is modified with a first labeling moiety, b) contacting the sample with an amount of a second purified antibody that specifically reacts with a second epitope of the extracellular ligand binding domain of sErbB1, wherein the second purified antibody is modified with a second labeling moiety, and wherein the second purified antibody does not competitively inhibit the binding of the first purified antibody, and c) determining presence or amount of the soluble epidermal growth factor receptor complexed with said antibodies by detecting the co-presence of the first and second labels. In preferred embodiments of this assay, the first antibody is either MAb R.1 or an antibody which binds to the same epitope as MAb R.1 (i.e. competitively inhibits the binding of MAb R.1 to the ligand binding domain of EGFR). In further preferred embodiments, the second antibody is MAb 528, or an antibody which binds to the same epitope as MAb 528 (i.e. competitively inhibits the binding of MAb 528 to the ligand binding domain of EGFR). In especially preferred embodiments, either the first or second labeling moiety is acridinium. In preferred embodiments of this aspect of the invention the patient biological sample

is blood, serum, plasma, urine, saliva, sputum, breast nipple aspirates, or ascites fluid.

Page 7, lines 12-22:

The invention further provides a diagnostic method for determining the presence, or risk, of an ovarian carcinoma in a female human patient. The method comprises a) determining the concentration of soluble EGFR in a biological sample obtained from a female patient with ovarian cancer (e.g., by the above immunochemical method,) b) comparing the concentration obtained in a) with a normal or baseline level for soluble EGFR that is preferably established with samples from female humans without ovarian cancer, and c) correlating a decrease in the concentration of sEGFR in the patient's sample with the presence of an ovarian carcinoma in the patient. In further embodiments of the aspect of the invention, a female patient may be monitored with repeated testing to determine the onset or progression of ovarian cancer. In further embodiments, the female patient may be tested before and after radiation, chemotherapy, or surgical treatment to monitor the regression or progression of ovarian cancer.

Page 7, lines 23-25, and Page 8, lines 1-4:

Yet another aspect of the invention is a method to increase or decrease the half-life of ErbB1 ligands in the circulatory system of a human patient. [In particular]For example, the method may be [uses]used to increase the circulatory half-life of ligands, such as EGF and TGF-α by binding to the ligands in the patient's blood, thereby inhibiting ligand degradation and extending the half-life of these ligands in the patient. Alternatively, the method may be used to decrease the circulatory half-life of these ligands by allowing cells to remove sErbB1-ligand complexes from the circulation by endocytosis and intravellular membrane transport. The method comprises administering to a human patient a ligand-half-life-[increasing]altering amount of a sErbB1 protein with an amino acid sequence chosen from the group consisting of SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, and SEQ ID NO. 6.

Page 8, lines 15-20:

Yet another embodiment of the invention is a method to regulate cellular proliferation and cellular differentiation. The sErbB1 molecules inhibit cytokines and receptors necessary for normal cell proliferation and differentiation and may play important roles in regulating development, wound healing, carcinogenesis, and tumor progression. The method comprises administering to a cell a cytokine-function-inhibiting amount of a sErbB1 protein with an amino acid sequence chosen from the group consisting of SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, and SEQ ID NO. 6.

Page 9, lines 8-9:

Figure 1. Schematic representation of full length, p60 sEGER, and normal p110 EGFR transcripts. Open boxes represent exons. Lines represent introns.

Page 9, lines 15-22:

Figure 5. Diagram showing the effects of p110 sErbB1 truncated protein *in vitro*. Chinese hamster ovary (CHO) cells were transfected with either the full-length human EGFR cDNA (encoding p170 EGER) in the expression vector pcDNA3 (Invitrogen) or with the vector alone. Stable clonal isolates were selected with G418 and these cells were then transiently transfected with the alternative 3.0 kb EGFR cDNA (encoding p110 sErbB1) or with vector alone. When p170 EGER and p110 sErbB1 were expressed in the same cells, significant cell death was observed 24-48 hours following transfection; however, no cell death was observed when either of these proteins were expressed individually.

Page 9, lines 23-25, and Page 10, lines 1-15:

Figure 6. This diagram illustrates the structure of several EGFR isoforms, including the full length EGF receptor, p170 ErbB1 (A), the mutant p110 soluble ErbB1 analog of A431 cells [which differs in sequence from normal p110 from human placenta, as described below] (B), the

p60 soluble ErbB1 analog of human placenta (C), and a recombinant p100 soluble ErbB1 analog (D). Additional information about these molecules is given in the chart. The full-length 170 kD ErbB1 receptor contains an extracellular domain with four distinct subdomains (I - IV), a transmembrane domain, and a cytoplasmic domain. The mutant A431 p110 sErbB1 analog contains extracellular subdomains I through IV and 17 unique amino acids at its carboxy-terminal end. The p60 sErbB1 analog contains subdomains I and II, a portion of subdomain III, and 2 unique carboxy-terminal amino acids. Recombinant human p100 sErbB1 ends at amino acid 589 (nucleotide numbering according to Ullrich *et al.*, 1984) and, therefore, embodies subdomains I through IV without any additional unique carboxy-terminal amino acids. The mutant p110 and 'natural' p60 human sErbB1 analogs are synthesized from truncated, alternatively [spliced.2.8]spliced 2.8 kb and 1.8 kb mRNA transcripts of A431 carcinoma cells and normal placenta, respectively. Qt6 cells transfected with the plasmid vector, psErbB1ECD589, [synthesizes]synthesize the recombinant human p100 sErbB1 analog. The p110 sEGFR encoded by the nucleic acids of the invention, not shown, contains subdomains I - IV, as well as its 78 unique amino acid carboxy end sequence.

Page 11, lines 9-10:

Figure 8. The concentration of sErbB1 in normal human female *(n[=40]=144)* and male *(n[=40]=88)* sera as measured by the ALISA of Example V are compared as described in Example VI.

Page 11, lines 17-21:

Figure 12. The serum sErbB1 concentrations, measured using the ALISA of Example V, of healthy women in a control group, with pre-operative [endothelial]epithelial ovarian cancer (EOC), with benign ovarian tumors, and with benign gynecological diseases are shown. The median sErbB1 concentration of women with EOC is significantly less than that of women without ovarian cancers.

Page 12, lines 22-25, and Page 13, lines 1-5:

As used herein, the term "soluble" epidermal growth factor receptor (sEGFR or sErbB1) means that the epidermal growth factor receptor polypeptide is found in a form [that is not anchored to the membrane of a cell]that does not harbor a transmembrane domain, *i.e.*, a portion of the sEGFR is not found physically embedded in the lipid bilayer which comprises the cell membrane in the [organsim]cell of its origin through a constituent peptide domain. However, SerbB1 may be embedded or attached to the cell membrane through other moieties such as lipids, carbohydrates, and/or proteins. Preferred soluble forms of the receptor are secreted by the cell [or proteolytically cleaved from the cell surface]proteolytically cleaved from the cell surface or released from the cell surface by other mechanisms. Other preferred soluble forms of the receptor may comprise the extracellular ligand binding domain of the [EGFR]sEGFR, and lack at least a portion of the transmembrane domain (TM), *i.e.*, the membrane-anchoring domain of the EGFR, so as to result in an EGFR which is not firmly anchored to, attached to, or embedded in the cell membrane.

As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a nucleic acid or polypeptide molecule from its natural or transformed cellular environment, and from association with other naturally occurring components of the cell. Such molecules may then be sequenced, replicated, manipulated, and/or recombined for artificial *in vivo* or *in vitro* expression. Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and is preferably substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source nucleic[,] acid with which it is normally associated" includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell.

As used herein, the term "[biologically]biological activity" of a peptide of the invention is

defined to mean a polypeptide comprising a subunit of a peptide having SEQ ID NO:1, or a variant thereof, which has at least about 10%, preferably at least about 50%, and more preferably at least about 90%, of the activity of a peptide having SEQ ID NO:1. The activity of a peptide of the invention can be measured by methods well known in the art including, but not limited to, the ability to bind EGF, or the ability of the peptide to elicit a sequence-specific immune response when the peptide is administered to an organism, *e.g.*, goat, sheep or [mice]mouse.

Page 14, lines 11-25:

As used herein, the term "synonymous," when used to describe nucleic acids, refers to the polypeptide sequence encoded by a compared nucleic acid relative to a reference nucleic acid. The genetic code is well known by those of skill in the art, and sequences with codon substitutions which encode the same amino acid can be easily devised for various purposes (e.g., introducing convenient restriction enzyme cleavage sites or optimizing codon usage for a particular recombinant protein production organism) without changing the translated polypeptide sequence. Utilizing common tools such as phosphoramidite polynucleotide synthesis and site directed mutagenesis or other recombinant techniques, such substitutions may easily be effected by those of ordinary skill in the art. Thus, sequences which are synonymous with the exemplary nucleotide sequences are also considered to be within the scope of the present invention. However, sequences which do not directly encode the translation of the same protein would not be considered to be "synonymous." Specifically, the full genomic [ErbB1]EGER/ERBB1 gene is not considered to be synonymous with the nucleic acids of the invention, as the full length gene can encode many alternatively spliced transcripts, including the nucleic acids of the invention, but the full-length transcript does not directly encode proteins such as SEQ ID NO. 1.

Page 16, lines 14-25:

The terms "transfected" or "transformed" are used herein to include any host cell or cell line, the genome of which has been altered or augmented by the presence of at least one

preselected DNA sequence, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "recombinant DNA," "exogenous DNA," "genetically engineered DNA," "non-native DNA," or "foreign DNA," wherein said DNA was isolated and introduced into the genome of the host cell or cell line by the process of genetic engineering. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence. Preferably, the transfected DNA is a chromosomally integrated recombinant DNA sequence, which comprises a gene encoding sEGFR, which the host cell may or may not express significant levels of autologous or "native" sEGFR.

Page 17, lines 3-14:

In order to more fully understand the mechanism by which the sEGFR influences cell growth and differentiation in normal tissue, cDNAs which encode the extracellular ligand binding domain, but not the cytoplasmic kinase domain, of the human epidermal growth factor receptor were isolated from a human placental cDNA library. Besides identifying a soluble form of the human epidermal growth factor receptor which may play a role in, or be associated with, cell growth and differentiation in normal cells, the identification and isolation of cDNAs encoding novel sEGFR transcripts has proven useful for defining the molecular basis for some neoplastic processes. Applicants have utilized monoclonal antibodies against the extracellular domain of EGFR to produce a useful diagnostic test which demonstrates the correlation between the discovered forms of sEGFR and ovarian cancer. By further utilizing the nucleic acid sequences of the invention to recombinantly produce the encoded sEGFRs, or producing specific antibodies to the unique carboxy terminal sequence of these proteins, other important advances in therapeutics and diagnostics may be realized through the invention.

Page 17, lines 15-21:

Applicants have demonstrated a significant correlation between the levels of sEGFR and

ovarian cancer. Thus, patient samples, *e.g.*, tissue biopsies, sera or plasma, may now be analyzed with antibodies specific for the sEGFR to detect the presence and progression of ovarian carcinomas in patients. As demonstrated by the examples below, applicants have found the level of sEGFR in patient samples to be useful in determining residual disease, responsiveness to chemotherapy, or overall survival. In addition, the levels of particular isotypes of sEGFR in a patient may be a useful indicator of the [histological]stage, grade, histological and molecular subtype of a tumor.

Page 17, lines 22-25, and Page 18, lines 1-3:

Furthermore, the cloning of transcripts encoding sEGFRs will elucidate the molecular mechanism giving rise to the presence or absence of sEGFR in patients with disease. Once the molecular mechanism underlying the expression of sEGFR is understood, molecular genetic based therapies directed to controlling the expression of sEGFR can then be employed to correct, inhibit or supplement the expression of sEGFR or full-length EGFR in patients with the disease. For example, an expression vector containing [DNA]cDNA encoding antisense sEGFR transcripts can be introduced into tumor cells to inhibit or reduce the overexpression of full-length EGFR.

Page 18, lines 6-15:

The cDNAs encoding sEGFR can also be employed in expression cassettes to synthesize sEGFR *in vitro*. *In vitro* prepared sEGFR can be employed to obtain antibodies specific for soluble forms of the EGFR. *In vitro* synthesized sEGFR may also be employed in a pharmaceutical formulation which, when administered to a human, can form [inactive] heterodimers with full-length [EGFR]ErbB family members to block [EGFR]receptor activation, compete with the full-length [EGFR]ErbB receptors for ligand, or block EGFR signal transduction post-ligand binding, and thus suppress or decrease the growth stimulatory [activity

of the EGFR]and other signaling activities of ErbB receptor tyrosine kinases.

Page 18, lines 18-24:

Sources of nucleotide sequences from which the present [DNA]cDNA molecules encoding human sEGFR can be derived include total or polyA$^+$ RNA from any human cellular source, preferably from embryonic cells such as those from placental tissue, carcinomas, or cell lines derived therefrom, from which cDNAs encoding sEGFR can be derived by methods known in the art and described below in Example I. Other sources of the DNA molecules of the invention include cDNA libraries derived from any human cellular source including placental cDNA libraries.

Page 21, lines 6-14:

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera *et al.*, Meth. Enzymol., 153, 3 (1987). Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook *et al.*, Molecular Cloning Laboratory Manual (Cold Spring Harbor Laboratory Press, N.Y. (1989)). Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard [technique]techniques.

Page 22, lines 3-12:

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutations(s). The

modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three [deoxyribonucleotidetriphosphates]deoxyribonucleotide triphosphates, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thiodeoxyribocytosine called dCTP-(aS) (Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

Page 25, lines 17-25:

sEGFR polypeptide expressed in a recombinant cell is purified from recombinant cell proteins or cellular polypeptides to obtain preparations that are substantially homogenous. For example, the culture medium or lysate can be centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. sEGFR polypeptide can then be purified from contaminant soluble or membrane proteins and polypeptides by fractionation on immunoaffinity or ion exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, *e.g.*, Sephadex G-75; or ligand affinity chromatography, and the like. An example of such purification is provided in Ilekis *et al.*, *supra*, as well as in Example II herein.

Page 29, lines 15-25:

[ ]It is preferred that the non-identical amino acids of engineered versions of these embodiments be conservatively substituted relative to the amino acids of SEQ ID NO. 1. It is

also preferred that the proteins of the invention have at least 10% of the biological activity of the polypeptide SEQ. ID NO. 1, more preferably at least 50% of the biological activity of the polypeptide SEQ. ID NO. 1, and most preferably at least 90% of the biological activity of the polypeptide SEQ. ID NO. 1. The activity of the sEGFR polypeptides of the invention can be measured by methods well known to the art including, but not limited to, ligand binding assays (Flickinger *et al.*, Mol. Cell. Biol., 12, 883 (1992)), the ability of the sEGFR to be bound by antibodies specific for the extracellular ligand binding domain of EGFR (*see* Example V, Maihle *et al.*, *supra* , and Ilekis *et al.*, *supra*), the ability of the sEGFR to inhibit the kinase activity of the full-length EGFR, and growth inhibition assays (*see* Example III). Amino acid substitutions are introduced into the DNA molecules of the invention by methods well known to the art. For example, see the description herein above for the introduction of silent mutations into the DNA molecules of the invention.

Page 30, lines 6-14:

Because the full amino acid sequence of the p110 sEGFR has been elucidated by the present invention, the invention also provides polypeptides useful for generating antibodies specific to the proteins encoded by the nucleic acid sequences of the invention, including SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, and SEQ ID NO. 6. By utilizing a portion of the sEGFR sequence which contains some of the 78 novel amino acids of the carboxy terminus of the disclosed [EGFR]sEGFR proteins, antibodies raised to these peptides may be specific for the placental p110 sEGFR, but non-reactive with full length EGFR or other sEGFR isoforms such as p60 sEGER. Thus, polyclonal or monoclonal antibodies particularly useful for detecting p110 sEGFR in patient samples may be made using the disclosed 78 amino acid

sequence.

Page 30, lines 15-25, and Page 31, line 1:

Polypeptides for use as immunogens will typically be smaller than the full sEGFR protein, ranging from 10 to about 500 amino acids in length. Although they may contain other amino acids for, e.g., conjugation purposes, the immunogenic polypeptides of the invention comprise an amino acid sequence specific for sEGFR which is of 10 to 25 amino acids in length, more preferably 11 to 21 amino acids in length, and most preferably 14 to 20 amino acids in length. These lengths of specific sequence are typical of those used in the art for conjugation to immunogenic carrier molecules. The amino acid sequence is identical to an amino acid sequence of similar length in an amino acid sequence selected from the group consisting of: amino acids 628-705 of SEQ ID NO. 1, amino acids 628-705 of SEQ ID NO. 4, amino acids 628-705 of SEQ ID NO. 5, and amino acids 628-705 of SEQ ID NO. 6. The polypeptides may optionally comprise further portions of the sEGFR amino acid sequence which [is]are not specific for the p110 sEGFRs, e.g., a peptide encoded by exon 15 and 15 b of sEGFR.

Page 31, lines 10-14:

After harvesting, monoclonal or polyclonal antibodies produced using the above polypeptides which are specific for [the] p110 EFGRs, and which do not cross-react with other ErbB1 isoforms, may be selected by screening for binding to recombinant p110 sEGER and p170 EGFR, as described in the Examples below.

Page 32, lines 21-25, and Page 33, lines 1-7:

In preferred embodiments of this aspect of the invention the patient biological sample is chosen form the group consisting of blood, serum, plasma, urine, saliva, sputum, breast nipple

aspirates, and ascites fluid. Especially preferred samples are serum, and plasma. [after]After incubation with the first antibody, the wells are rinsed, and then the second labeled antibody is added. In this format, the second labeling moiety is a detectable labeling moiety such as a fluorescent, colorigenic, or chemiluminescent moiety. A preferred moiety for use as the second labeling moiety is acridinium, as the applicants have found its strong signal useful in determining especially low concentrations (femtomolar range). After incubation with the second antibody, the wells are again rinsed. Proper reaction components are then added, and the second labeling moiety detected by fluorometry, colorimetry, or luminometry. Thus, the co-presence of the two labels is determined by their location (attached to the well) and their detectable product (fluorescence, light, or colorimetric product).

Page 34, lines 6-9:

The results presented [below] show that ovarian cancer patients with survival times twice that of the mean survival times of patients with ovarian cancer had detectable levels of sEGFR while patients with average, or less than average, survival times had undetectable levels of sEGFR. Thus, the absence, or low levels, of sEGFR may be indicative of aggressive disease.

Page 34, lines 18-23:

Isolation and Characterization of Human [*c*-erbB1]EGFR/ERBB1 cDNAs Encoding Soluble EGFRs

To isolate human EGFR clones that lack sequences encoding the cytoplasmic domain, but have the extracellular domain, differential hybridization was employed to screen an oligo-dT primed human placental cDNA library (Clontech, cat. # H1144x). The library was screened for clones that were positive for a ligand binding domain (LBD) specific probe (positions 174-2105), but negative for a kinase domain (KD) probe (see Figure [1]2 for full length/ p110 comparison).

Page 34, lines 24-25, and Page 35, lines 1-11:

The ligand binding domain probe was synthesized by the PCR using pXER as a template (Chen *et al.*, Nature , 32, 820 (1987)). The forward primer was: SEQ ID NO: 7, corresponding to nucleotide positions 174-193. The reverse primer had the sequence SEQ ID NO:8, representing base pairs [1986]2086-2105. Nucleotide numbering is according to Ullrich *et al.*, *supra*, unless stated otherwise. Amplification was performed for 35 cycles (94° C for 1 minute; 65° C for 1 minute; 72° C for 3 minutes) with a final extension at 72° C for 10 minutes. The PCR product was then excised from a low melting point agarose gel. A 768 bp EcoRI fragment from pXER was gel purified and used as the intracellular kinase domain (KD) probe. The LBD and KD probes were radiolabeled with [α-^{32}P]dCTP using a random primer DNA labeling kit (Gibco BRL) according to the manufacturer's instructions. The hybridizations were performed in a solution containing 6X SSC, 5X Denhard's, 7.5% dextran sulfate, 0.5% N-lauryl sarcosine, and 100 μg/ml salmon sperm DNA at 65° C. Filters were washed in 0.1X SSC and 0.1% N-lauryl sarcosine at 65° C and then were exposed to x-ray film for 24 to 72 hours at -80° C with an intensifying screen.

Page 35, lines 17-25, and Page 36, lines 1-3:

To determine whether the transcript [represented]encoding by p110 [cDNA]sEGFR is expressed in human placenta, RNA from a human placenta cell line (ATCC, CRL 1584) was isolated by a guanidine isothiocyanate procedure. Isolated RNA was treated with RNase free DNase and extracted twice with 1:1 phenol:chloroform. RNA (1 μg) was heated to 90° C for 5 minutes, then the RNA was reverse transcribed in a 20 μl reaction containing 1X Avian

Myeloblastosis Virus (AMV) reaction buffer, 1 [MM]mM each dNTP, 10 mM dithiothreitol (DTT), 20 U RNAsin, 10 U AMV reverse transcriptase, and 0.1 μg oligo-dT at 24° C for 10 minutes, 42° C for 50 minutes, 99° C for 5 minutes and then 4° C for 5 minutes. The first strand cDNAs were then amplified by adding Taq polymerase to the reverse transcription reaction along with [EX15F and EX15R]pEXI5F (SEQ ID NO:9) and pEXI5BR (SEQ ID NO:13) in a final volume of 100 μl under the amplification conditions described hereinabove. The amplified products were analyzed by 5% PAGE. The results show that the [RNA represented by]3.0 kb transcript is expressed in the human placenta cell line.

Page 36, lines 4-14:

Thus, the isolated clone represents a 3.0 kb alternative transcript of EGFR. To map the 3.0 kb transcript, the following primers were employed: P1981 (EX15F; SEQ ID NO:9), P267F (EX15bF; SEQ ID NO:10), P615F (EX15bF; SEQ ID NO:11), P297R (EX 15bR; SEQ ID NO:12) and P732R (EX15bR; SEQ ID NO:13). The 3.0kb transcript arises from an alternative splicing event from exon 15 to a novel exon located within intron 15 of the EGFR gene. This novel exon contains 2 polyadenylation sites. None of the downstream EGFR exons are included in this transcript. This transcript differs from the 2.8 kb transcript unique to A431 cells as the A431 transcript contains EGFR exons 1 to 16 and then splices to an unrelated sequence derived from a translocation. The 3.0 kb transcript encodes a polypeptide of 681 amino acids (less the 24 amino acid signal peptide) containing 78 unique carboxy-terminal [amino][acids,] amino acids Pro 628- His 705 of SEQ ID NO. 1.

Page 36, line 24:

Soluble Human [*c*-erbB1]EGFR/ERBB1 Gene Product

Page 36, line 25, and Page 37, lines 1-10:

The amino acid sequence deduced from the 3.0 kb [transcript]EGFR/ERBB1 cDNA, (SEQ ID NO:1), predicted a [681]705 amino acid polypeptide [after cleavage of the signal peptide] with a molecular mass of 77 kD. The first 24 amino acids code for a signal peptide; following cleavage by signal peptidases, the predicted molecular weight of this polypeptide is 75 kD. The sequence encodes subdomains I, II, III and a portion of subdomain IV of the extracellular ligand binding domain of the EGFR plus an additional 78 unique carboxy-terminal amino acids. A quail fibroblast cell line, QT6, was transiently transfected with the plasmid pDR2241, which contains the 3.0 kb [sErbB1]EGER/ERBB1 transcript and [encodes]synthesizes a 110 kD [polypeptide (p110) that has ErbB1 ligand binding subdomains I through IV plus an additional 78 unique carboxy-terminal amino acids]glycosylated polypeptide (p110 sErbB1). Cells were transfected with 15 µg of pDR2241 by the calcium phosphate precipitation technique as described previously (Ausubel *et al.*, [Curren]Current Protocols in Molecular Biology, John Wiley & Sons, NY (1994)).

Page 37, lines 11-23:

Transfected cells from two 10 cm plates were pooled and replated in 6 well plates approximately 48 hours post-transfection. The following day, cells were [reused]rinsed once in phosphate buffered saline (PBS) and labeled in methionine free DMEM supplemented with 5% dialyzed FCS and 150 [gCi]µCI/ml of [^{35}S][^{35}S] methionine (Promix, Amersham) at 37° C for 12 hours. Conditioned medium from labeled cells was collected and centrifuged briefly to remove loose cells and debris and phenylmethylsulfonyl fluoride (PMSF) and aprotinin were added to a final concentration of 1 mM and 50 µg/ml. Cell monolayers were lysed and immunoprecipitated

with the addition of 1 to 5 μg of monoclonal antibody as described previously (Maihle *et al.*, Mol. Cell. Biol., 8, 4868 (1988)). Samples were resuspended in 2X Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 2 mM EDTA, 0.04% bromphenol blue), boiled for 5 minutes and separated by 10% SDS-PAGE. Gels were stained with Coomassie blue, treated with EnHance (Dupont) and dried before an overnight exposure to x-ray film.

Page 37, lines 24-25, and Page 38, lines 1-6:

Immunoprecipitation of mock transfected cells failed to reveal a specific EGFR related polypeptide in either cell lysates or in conditioned media, while a 115 kD soluble EGFR was immunoprecipitated from the media of control A431 cells. [As predicted, i]Immunoprecipitation of cell lysates from transfected cells revealed a heterogeneous 110 kD species that was specifically recognized by the EGFR specific monoclonal antibody, R1 (Amersham, RPN 513) (Waterfield *et al.*, J. Cell. Biochem., 20, 149 (1989)). Thus, expression of the nucleic acids of the invention encoding soluble truncated forms of the human EGFR results in a glycosylated sEGFR protein when transgenically expressed in [mammalian]eukaryotic cells.

Page 38, line 11:

Growth inhibitory Potential of Soluble [*c-erbB1*]ErbB1 Receptors on Ovarian Carcinoma Cell Growth *in Vitro*

Page 38, lines 27-28, and Page 39, lines 1-18:

To determine the function of the human 3.0 kb alternative transcript which [produces]encodes p110 sErbB1, [we used]a quantitative ribonuclease protection assay was used to determine its relative abundance in RNA from 17 adult (brain, breast, colon, heart, kidney, liver, lung, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis,

thymus, and uterus) and 4 fetal tissues (brain, kidney, liver, and lung), as well as in numerous carcinoma-derived cell lines, either with or without *EGFR* gene amplification. The riboprobe consisted of 313 nt (1754-2066 in X00588) shared by both the full-length and the 3.0 kb transcripts, plus 134 bp of exon 15B which was specific for the 3.0 kb mRNA. Transcripts containing exon 15B would result in a protected fragment of 447 nts, whereas those containing exon 15 spliced to exon 16 would result in a protected fragment of 313 nts. The full-length transcripts were observed in all of the samples examined, while the 3.0 kb transcript was detected only in human placenta, and in the carcinoma cell lines which also contained amplification of the *EGFR* gene [(Figure 7A)]. The relative ratio of full-length to 3.0 kb transcripts was quantified using a phosphoimager. The relative level of the full-length mRNA was ~200-fold greater than the level of the 3.0 kb transcript in both human placenta and in the MDA-MB-468 breast carcinoma cell line, which contains ~15-fold amplification of the *EGFR* gene (Filmus *et al.*, 1985). However, in the A431 carcinoma cell line containing ~30-fold amplification of the *EGFR* gene, the full-length transcript was only present in 100-fold excess compared to the 3.0 kb mRNA. The 3.0 kb transcript was not detected in the absence of *EGFR* gene amplification in other carcinoma-derived cell lines.

Page 39, lines 19-26:

Normal cells are expected to tolerate co-expression of p110 sErbB1 and EGFR because these proteins may route to distinct membranes (i.e., apical *vs.* basolateral), [and that]whereas co-localization of these receptors [will]may result in apoptosis. Accordingly, the loss of cell polarity/membrane organization, which is characteristic of high-grade carcinomas, [results]may result in selection against p110 sErbB1 expression in ovarian tumors (our preliminary results

suggest that this is, indeed, the case). As p170 ErbB1 expression is usually up-regulated in ovarian cancer cells as compared to p110 sErbB1, increased exposure to p110 sErbB1 is expected to prove toxic to these cells. Thus, the p110 sEGFR may be used to produce apoptosis in ovarian cancer cells:

Page 40, lines 6-8:

[Cultures of ovarian carcinoma cells exposed to sEGFR preparations have reduced growth rates compared to cells which are not exposted to sEGFR. Thus, sEGFR can inhibit carcinoma cell proliferation.]

Page 41, lines 1-10:

To prepare monoclonal antibodies [(Mab)](MAbs) specific for epitopes present in domains I, III or IV of full-length human EGFR, synthetic peptides were prepared. The peptides were predicted to have a high hydrophilicity, surface probability, and antigenicity. The peptides correspond to amino acids 77 to 93 of subdomain I (SEQ ID NO:14), 290-311 of subdomain III (SEQ ID NO:15), 352-369 of subdomain III (SEQ ID NO:16), and 556-567 of subdomain IV (SEQ ID NO:17) of the [compete]complete ErbB1 receptor, which lacks the signal peptide. Peptide immunogens were made by coupling the peptides to keyhole limpet hemocyanin and bovine serum albumin. The maleimide coupling chemistry required the addition of cysteines to the c-terminus of the 77 to 93 and 352-369 peptides, while the native cysteines of the 290-311 and 556-567 peptides were used for coupling.

Page 41, lines 11-21:

Although every immunized mouse elicited an immunological response toward its cognate peptide, as determined by ELISA, only mice injected with peptides having SEQ ID NO:15, SEQ

ID NO:16 or SEQ ID NO:17 produced antibodies capable of recognizing the 170 kD ErbB1 receptor (p170) from A431 whole cell lysates by Western blot. Hybridomas from mice injected with peptides having amino acids 290 to 311 (clone 10B7), 352-369 (clones 15E11 and 17H3), and 556-567 (clone 2D2) of the mature EGFR were generated. All of these clones produced monoclonal antibodies [(MAbs)] that recognized p 170 ErbB1 in Western blots, and that bound to A431 cells as detected by immunofluorescence microscopy. Two MAbs (15E11 and 2D2) were also compatible with immunohistochemical methods, whereby cells or tissues are embedded in paraffin, sectioned, treated with steam and citrate to retrieve masked antigens, immunolabeled, and processed to visualize antigen with diaminobenzidine.

Page 41, lines 22-25:

Three MAbs (10B7, 15E11, and 2D2) were able to immunoprecipitate p170 ErbB1 from whole cell lysates of A431 cells. One of these MAbs (15E11) immunoprecipitated a 60 kD soluble form of human [ErbB1]sErbB1 (p60) from whole cell lysates and culture media of transfected QT6 quail fibroblasts (QT6/pDR161). MAb 15E11 also detected p60 [ErbB1]sErbB1 in QT6/pDR161 culture media by ALISA (acridinium-linked immunosorbent assay). MAb 15E11 was covalently coupled to Protein G and this resin was employed to purify (80% homogeneity) p60 ErbB1 from QT6/pDR161 culture media by immunoaffinity chromatography. This MAb reacted in the same manner to p110 ErbB1.

Page 42, lines 5-7:

Hybridoma clones 10B7, 15E11, 17H3 and 2D2 have been deposited with the American Type Culture Collection, in accord with the requirements of the Budapest Treaty, and granted Accession Nos. [____, ____, ____, and ____,]HB-12204, HB-12205, HB-12206, and HB-12207,

respectively.

Page 43, lines 5-11:

A sensitive acridinium-linked immunosorbent assay (ALISA) to quantify sErbB1 and ErbB1 molecules in patient body fluids and tissues, respectively, was developed by applicants. This ALISA as described below was used to quantify serum sErbB1 levels in healthy men and women, as further described in Example VI, [as well as to demonstrate that serum samples of healthy men and women contain a sErbB1 analog of approximately 110 kD (See Figure 14.)]and in patients with ovarian cancer. The experimental results of Baron et al., J. Immunol. Methods, 219, 23 (1998) are incorporated by reference. The ALISA was developed using the following:

Page 44, lines 14-25, and Page 45, lines 1-17:

A cDNA fragment encoding 589 amino acids of the human ErbB1 receptor's ECD was subcloned from pXER (Chen *et al.*, 1989; a.k.a. pXEGFR, Opresko *et al.*, 1995) into pcDNA3 (Invitrogen, Carlsbad, CA). pXER was digested with Nae I; an approximately 3.5 kb cDNA fragment was resolved by agarose gel electrophoresis and gel purified using GENECLEAN™ (BIO 101, La Jolla, CA). The 5'- Nae I restriction enzyme site was located within the pXER vector, whereas the 3'- Nae I site was positioned just before nucleotide 2026 of the [*c*-erbB1]EGFR/ERBB1 cDNA; the nucleotide numbering system is that of Ullrich *et al.* (1984). Xba I linkers (Boehringer Mannheim, Indianapolis, IN) were phosphorylated with T4 polynucleotide kinase and ligated to the blunt-ended approximately 3.5 kb Nae I restriction fragment with T4 DNA ligase. The linker-ligated restriction fragment was subsequently digested with Xba I to yield two DNA fragments of approximately 1.5 kb and approximately 2.0 kb. The larger approximately 2.0 kb [*c*-erbB]EGFR/ERBB1 cDNA fragment encoding the ECD of ErbB1

was purified as described above and ligated to Xba I digested, calf intestine phosphatase treated pcDNA3. *E. coli* DH5-α were transformed with pcDNA3 constructs containing the approximately 2.0 kb [*c*-erbB1]EGFR/ERBB1 cDNA according to the method of *Hanahan* (Maniatis *et al.*, 1982) and grown on Luria-Bertani agar plates containing 100 μg/ml ampicillin as a selectable marker. Plasmid DNA was isolated by the boiling method from transformed bacterial colonies (Ausubel *et al.*, 1989) and digested with BamH I to determine the orientation of the approximately 2 kb [*c*-erbB1]EGFR/ERBB1 *insert* in pcDNA3. Plasmids containing the approximately 2 kb [*c*-erbB1]EGFR/ERBB1 fragment in the sense orientation yield a BamH I restriction fragment of 1348 bp, whereas clones containing the approximately 2 kb [*c*-erbB1]EGFR/ERBB1 fragment in the antisense orientation yield a 678 bp restriction fragment. A transformed clone of *E. coli* DH5-α containing the approximately 2.0 kb [*c*-erbB1]EGFR/ERBB1 fragment in the sense orientation was identified and plasmid DNA, called psErbB1ECD589, was prepared using the QIAGEN plasmid purification kit (QIAGEN, Chatsworth, CA) for transfection experiments. The quail fibroblast cell line, Qt6, was subsequently transfected with psErbB1ECD589 by calcium phosphate precipitation (Wigler *et al.*, 1979) and stable, geneticin [(648)](G418) resistant cells were isolated and cloned by limiting dilution. A clonal cell line, Qt6/psErbB1ECD589, expressing p100 sErbB1 was identified by immunoprecipitation of ^{35}S-labeled cell lysates with MAb C11 using methods described previously (Maihle *et al.*, 1988).

Page 46, lines 6-22:

Tissue culture cells at 80% to 90% confluence were rinsed once with phosphate buffered saline (PBS; 10 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl, pH 7.2), scraped from the PBS loaded

petri dish with a cell lifter (Costar, Cambridge, MA), and harvested by centrifugation at approximately 1000X g for 5 minutes. The cell pellet was resuspended and lysed by adding a 1:10 (w/v) ratio of membrane protein immunoprecipitation lysis buffer containing protease inhibitors (10 mM Trizma®, pH [7.4,150]7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 150 µg/ml phenylmethyl sulfonyl fluoride, 2 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin A). The cell lysate was vigorously vortexed for 30 seconds and sonicated (three 10 second bursts at half power with 60 second cooling periods) with a sonicator (model W-225R; Heat Systems-Ultrasonics, Farmingdale, NY) to break DNA molecules. Enough 5.0 M NaCl was added to the cell lysate to bring the final NaCl concentration to 500 mM. Cellular debris was pelleted by centrifugation at 10,000X g for 10 minutes. The resulting supernatant was exchanged into Trizma buffered saline (TBS; 10 mM Trizma, pH 7.4, 150 mM NaCl) containing 0.02% NaN_3 by passage through a Sephadex G-25 fast desalting gel filtration column using a Fast Performance Liquid Chromatography system [(FPLCO](FPLC; Pharmacia Biotech, Piscataway, NJ), and concentrated by ultrafiltration with a Centricon centrifugal concentrator (Amicon, Beverly, MA). The protein concentration of the final whole cell lysate was determined by BCA (Pierce Chemical).

Page 46, line 25, and Page 47, lines 1-15:

A 1 mg/ml stock solution of succinimidyl-activated acridinium ester [4-(2-succinimidyl-xycarbonylethyl)phenyl-10-acridinium-9-carboxylate sulfonate; ASSAY Designs, Ann Arbor, MI) in dry dimethyl formamide was stored in 5 µg aliquots at -70° C. MAb IgG was exchanged from carrier solution into labeling buffer (0.2 M sodium phosphate buffer (NaH_2PO_4/Na_2HPO_4) pH 8.0) with a Sephadex G-25 fast desalting gel filtration column by [FPLCO]FPLC and concentrated by

ultrafiltration to 200 µl and approximately 1.0 mg/ml total protein. MAb IgG was labeled at room temperature with a 1:80 molar ratio of IgG to succinimidyl-activated acridinium ester for 15 minutes in the dark. The coupling reaction was stopped by adding 100 µl of quenching buffer (labeling buffer with 10 mg/ml lysine monohydrochloride) and incubating for an additional 5 minutes. Unbound acridinium ester was removed with a Sephadex® G-25 fast desalting gel filtration column by [FPLCO]EPLC and simultaneously exchanged into a solution containing 0.2 M sodium phosphate buffer, pH 7.3, and 0.02% NaN_3. Following buffer exchange by [FPLCO]EPLC, the acridinium-labeled MAb IgG was concentrated by ultrafiltration to a volume of 100 µl; 1.0 ml of storage buffer (0.2 M sodium phosphate buffer, pH 7.3, 0.1% bovine serum albumin, 0.02% NaN_3) was added and the volume was reduced again to 100 µl. The Relative Light Units (RLU)/ µl were determined and the final acridinium-labeled MAb IgG was stored at -70° C.

Page 47, lines 18-25, and Page 48, lines 1-14:

White XENOBIND® 96 well microtiter plates (Xenopore, Saddle Brook, NJ) were coated overnight at 4° C with 25 µg/well of an affinity-purified goat anti-mouse IgG_{2b} specific polyclonal antibody in carbonate buffer (90 mM [$NaHCO_3$)]$NaHCO_3$ 10 mM Na_2CO_3, pH 9.4, 0.02% NaN_3); the antigen is attached covalently to the plate under these conditions. All incubations were performed on a rocker platform. The plates were washed three times with high salt Tween-20® wash buffer (HST-20WB; 0.05% polyoxyethylene sorbitan monolaurate [[Tween-20®,](Tween-20®, 20 mM Trizma®, pH 7.4, 500 mM NaCl, 0.02% NaN_3) blocked with ALISA blocking buffer (ALBB; 2.0% bovine serum albumin (BSA), 10 mM Trizma pH 7.4, 150 mM NaCl, 0.02% NaN_3) for 1 hour at room temperature, washed with HST-20WB,

incubated with 0.05 μg/well anti-ErbB1 ECD-specific MAb R.1 diluted in ALBB for 2 hours at 37° C, washed three times with HST-20WB, incubated with analyte or unknown sample for 2 h at 37° C, washed three times with HST-20WB, incubated with acridinium-labeled anti-ErbB1 ECD-specific MAb 528 (500,000 counts/well) for 1 hour at 37° C, washed three times with HST-20WB, and read with a luminometer (model LB 96P; EG&G Berthold Analytical Instruments, Nashua, NH). In order to maintain an even temperature across the microtiter plate, all 37° C incubations were performed in a forced-air environmental shaker (model 3528; Lab-Line Instruments, Melrose Park, IL) that was modified to hold microtiter plates. Acridinium decomposition was initiated by sequentially adding a solution containing 0.441% nitric acid and 0.495% H_2O_2 followed by a solution containing 0.25 M NaOH and 0.1875% cetyltrimethylammonium chloride. This treatment drives the acridinium ester to form an unstable dioxetanone intermediate, which decomposes to form N-methylacridone in its excited singlet state; relaxation to the ground state results in the emission of photons of light at a wavelength of 430 nm (Weeks *et al.*, 1986).

Page 48, lines 24-25, and Page 49, lines 1-3,:

As shown in Figure 7, the above ALISA is specific for molecules containing subdomain IV of the extracellular domain of ErbB1 (p170 ErbB1, A431 mutant p110 sErbB1, recombinant p100 sErbB1), but does not bind to molecules which do not contain [the subdomain]subdomain IV (p60 sErbB1 and other unrelated molecules). Also, as demonstrated in this and the following examples, the ALISA is highly sensitive, and can detect sErbB1 in the femtomolar range.

Page 49, line 5:

Immunoprecipitation and Characterization of sErbB1 analogs from normal human sera [and

Characterization]

Page 49, lines 19-25, and Page 50, lines 1-11:

Normal human sera were cleared of lipids with Seroclear® according to the manufacturer's protocol (Calbiochem-Novabiochem, La Jolla, CA), diluted 1:5 (v/v) in Affi-Gel Protein-A MAPs II® binding buffer (Bio-Rad Laboratories, Hercules, CA) and clarified of human IgG molecules by passage through a Protein-G Superose® affinity column by [FPLC®]FPLC using Affi-Gel Protein-A MAPs II® binding, elution, and regeneration buffers (Bio-Rad Laboratories). The column flow through was collected, concentrated by ultrafiltration, exchanged into TBS containing 0.02% NaN_3 by [FPLC®]FPLC with a Sephadex® G-25 fast desalting gel filtration column, divided into four aliquots, and immediately incubated with uncoupled (MAb minus) or MAb R.1-, 225-, and 528-coupled affinity resins. Alternatively, human serum samples were simply clarified of human IgG molecules by incubating with Immunopure® Immobilized Protein-G resin (Pierce Chemical) for 30 minutes at room temperature prior to immunoprecipitation. Both methods yielded identical immunoprecipitation results. The MAb R.1, 225, and 528 affinity resins were prepared with an Immunopure® Immobilized Protein-G IgG Orientation kit according to the manufacturer's instructions (Pierce Chemical); the MAb minus resin was prepared in an identical manner, except that no IgG was bound to Protein-G. Bound proteins were eluted from these resins with MAPs II® elution buffer, adjusted to neutral pH by adding 1.0 M Trizma® buffer (pH 9.0), and analyzed by SDS-PAGE and Western immunoblot.

Page 51, lines 18-21:

As shown in Figure 14, a 110 kD protein was immunoprecipitated from normal human male and female sera with the ECD-specific anti ErbB1 antibodies used in the above [ALISA, believed to be the same 110 kD protein isolated from a human placental cDNA library as described above and is comprised of the 110 kD p110 sErbB1 SEQ ID NO. 2, and its variants.]ALISA. Microsequence analysis of partially pure p110 sErbB1 from human serum using Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry shows that this protein is derived from the 3.0 kb alternative transcript having SEQ ID NO. 2 of the invention.

Page 52, lines 11-15:

Results of these experiments are shown in Figure 15. Although the Oncogene ELISA did react with some substance in the sera of healthy men and women, it did not detect any difference in sErbB1 concentration between the two, nor did the ELISA results correlate with the applicants' ALISA results. Thus, the commercially available ELISA test [appers to be less sensitive and accurate than the]differs substantially from the ALISA described herein.

Page 53, lines 6-18:

Medical records were reviewed systematically to ascertain menopausal status at the time of the blood draw. Data collected include: date and patient age at the time of blood draw, date of last menstrual period (LMP), self-reported symptoms of menopause, date of surgical menopause (hysterectomy and/or oophorectomy), and FSH and/or LH levels. For patients who underwent surgical hysterectomy but not oophorectomy, the clinical records also were reviewed to determine the approximate date of clinical menopause (patient's self-report). The criteria used to assign menopausal status were one or more of the following: 1) age $\geq$ [00]60 years, 2) last reported menstrual period > six months from the date of the blood draw, 3) symptoms of menopause, 4)

hysterectomy, 5) oophorectomy, 6) FSH level [< or > 00 IU/L, and LH level < or > 00 IU/L]≤30 IU/L (premenopause) or ≥36 IU/L (postmenopause). Menopausal status for 6 of the 144 women could not be determined in this study, because a) FSH levels were equivocal or there was insufficient serum to measure these [levels, b)]levels, and b) the patient's medical record was incomplete; i.e., a long time interval between the patient's last medical visit and the blood draw or the patient transferred health care to another [facility)]facility.

Page 55, lines 5-19:

To explore the diagnostic potential of sErbB1, applicants utilized the ALISA described in Example V to compare pre-operative serum sErbB1 levels between 149 healthy women, 164 stage I, II, III, or IV ovarian cancer patients, 142 patients with benign ovarian tumors, and 115 patients with other benign gynecologic diseases of the pelvis. The individuals in each of these cohorts are within the same age range, but are not age-matched on a one-to-one basis. The cohort of patients with benign ovarian tumors had the following diagnoses: simple cyst, corpus luteum cyst, follicular cyst, dermoid, fibroma, mucinous cystadenoma, and serous cystadenoma; and the cohort of patients with other benign gynecologic pelvic diseases had the following diagnoses: paratubal cyst, cervical dysplasia, endometriosis, fibroids, and hydrosalpinx. [This]The scattergram in Figure 12 clearly shows that serum sErbB1 levels in EOC patients are significantly lower than those seen in healthy women (non-surgical patients), in patients with benign ovarian tumors, and in patients with other benign gynecologic pelvic diseases. Moreover, these data indicate that pre-operative serum sErbB1 levels may be useful in making a diagnosis between early, as well as late stage EOC versus benign ovarian tumor and other benign gynecologic pelvic disease.

Page 56, lines 18-22:

Following collection, all blood samples were allowed to clot at room temperature for 30 min. The serum was separated from the clot and cells by centrifugation at 2000 ~ X *g* for [10 min.]10 min., divided into 1-ml aliquots, and stored at -70°C. Each serum sample was thawed after transfer into our laboratory, aliquoted into smaller volumes, and refrozen at -70°C to prevent sErbB1 and EGF degradation. Each serum sample was, therefore, frozen and thawed only twice.

Page 58, lines 24-27, and Page 59, lines 1-11:

Research suggests that p110 sErbB1 is localized to the membrane via the addition of a glycosylphosphatidylinositol (GPI) anchor. The core structure common to all GPI anchors consists of a glycan bridge between phosphatidylinositol and phosphoethanolamine with phosphoethanolamine attached to the carboxy-terminus of the protein. GPI anchors are added to proteins containing an appropriate carboxy-terminal signal sequence. This linkage is a post-translational modification that occurs in the lumen of the endoplasmic reticulum within minutes of protein synthesis. The carboxy-terminal signal sequence consists of a cleavage/attachment site, called the omega site [(ù)](ω), a short hinge region that contains charged amino acids, and a carboxy-terminal hydrophobic region of varying length. The carboxy-terminal signal sequences of several known GPI proteins, as well as the predicted signal sequences present in human p110 sErbB1 (and in an analogous mouse p110 sErbB1) are listed in [Table 4]Table 2. Compared to known GPI signal sequences, both the human (SEQ ID NO:18; SEQ ID NO:19) and the murine (SEQ ID NO:20) sErbB1 receptors contain potential carboxy-terminal GPI anchor signal sequences. In fact, the human p110 sErbB1 product contains 2 putative signal sequences; the significance of tandem signal sequences is not known, but potentially either could be used.

Page 60, line 18:

<130> [Atairgin]~~TBIG~~ 257/225

MARKED UP AMENDED CLAIMS:

9. (Once Amended) An assay for determining the concentration of soluble epidermal growth factor receptor and full-length epidermal growth factor receptor in a biological sample from a human patient, the assay comprising:

a) obtaining a biological sample from the patient;

b) contacting an amount of a first purified antibody that specifically reacts with a first epitope of the extracellular ligand binding domain of sErbB1 with the patient biological sample to be tested, wherein the first purified antibody is modified with a first labeling moiety;

c) contacting the sample with an amount of a second purified antibody that specifically reacts with a second epitope of the extracellular ligand binding domain of sErbB1, wherein the second purified antibody is modified with a second labeling moiety, and wherein the second purified antibody does not competitively inhibit the binding of the first purified antibody; and

d) detecting the co-presence of the first and second labels to determine the concentration of the soluble epidermal growth factor receptor complexed with the antibodies; wherein one of the antibodies is chosen from the group consisting of: M[a]Ab R.1 and antibodies which competitively inhibit the binding of M[a]Ab R.1 to ErbB1; and wherein the other antibody is chosen from the group consisting of M[a]Ab 528 and antibodies which competitively inhibit the binding of M[a]Ab 528 to ErbB1.

11. (Once Amended) The assay of Claim [11]9 wherein the patient biological sample is chosen from the group consisting of blood, serum and plasma.